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Absolute MR thermometry using nanocarriers

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Accurate time-resolved temperature mapping is crucial for the safe use of hyperthermia-mediated drug delivery. We here propose a magnetic resonance imaging temperature mapping method in which drug delivery systems serve not only to improve tumor targeting, but also as an accurate and absolute nano-thermometer. This method is based on the temperature-dependent chemical shift difference between water protons and the protons in different groups of drug delivery systems. We show that the chemical shift of the protons in the ethylene oxide group in polyethylene glycol (PEG) is temperature-independent, whereas the proton resonance of water decreases with increasing temperature. The frequency difference between both resonances is linear and does not depend on pH and physiological salt conditions. In addition, we show that the proton resonance of the methyl group in *N*-(2-hydroxypropyl)-methacrylamide (HPMA) is temperature-independent. Therefore, PEGylated liposomes, polymeric mPEG-b-PPHMA-Lac₂ micelles and HPMA copolymers can provide a temperature-independent reference frequency for absolute magnetic resonance (MR) thermometry. Subsequently, we show that multigradient echo MR imaging with PEGylated liposomes *in situ* allows accurate, time-resolved temperature mapping. In conclusion, nanocarrier materials may serve as highly versatile tools for tumor-targeted drug delivery, acting not only as hyperthermia-responsive drug delivery systems, but also as accurate and precise nano-thermometers. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: drug delivery; liposomes; micelles; MR thermometry; polymers

1. INTRODUCTION

Drug delivery systems, such as liposomes, are designed to improve the biodistribution of systemically administered (chemo-) therapeutic agents, and to thereby increase the efficacy and reduce the toxicity associated with the therapy (1). The entrapment of drugs within liposomes prolongs their circulation time, especially when the liposomes are surface-modified with polyethylene glycol (PEG) (2). This prolonged circulation time enhances the accumulation of drugs in tumors and sites of inflammation via the enhanced permeability and retention (EPR) effect (3). There is growing evidence that mild hyperthermia can be used to potentiate drug targeting to tumors, either by enhancing the EPR-mediated extravasation of liposomes, polymers and micelles from the tumor vasculature, or by triggering drug release from thermosensitive liposomes (4–6). In both cases, however, there is a delicate balance between improving tumor-directed drug delivery and drug release on the one hand, and shutting down tumor blood flow and causing thermal damage to surrounding healthy tissues on the other. Therefore, there is a need for accurate and time-resolved temperature mapping in order to control the heating of tissues by external heat sources like laser, radiofrequency fields and high-intensity focused ultrasound (HIFU).

Magnetic resonance imaging (MRI) is a medical imaging modality that offers possibilities for noninvasive imaging of temperature distributions inside human tissue. MR thermometry is currently routinely used in the clinic for fast and accurate temperature mapping during HIFU ablations of uterine fibroids (7,8). In addition, MR-guided HIFU holds great promise for controlled hyperthermia-assisted local drug delivery (9–11). Several different MRI-based methods have been proposed for performing thermometry, based on the temperature-dependence of parameters

that influence the measured MR signals, such as the proton resonance frequency (PRF), the longitudinal relaxation time (T_1) and the diffusion constant of water molecules (D) (12,13). These methods are generally used to detect temperature differences, implying the need for a known reference temperature if absolute thermometry is required. *In vivo*, however, the noninvasive determination of an absolute reference temperature is difficult.

To date, the most commonly used method for performing absolute MR thermometry that does not require a known reference temperature is based on exploiting the temperature-dependent

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changes in the electron screening constant of water protons leading to temperature-dependent chemical shift changes of the water proton resonance relative to that of a reference resonance that does not shift as a function of temperature. The resulting resonance frequency difference is linearly related to temperature, and can be measured in a single voxel using MR spectroscopy (MRS) (14,15). Absolute temperature mapping, that is, creating temperature images of the tissue, can be achieved using MR imaging techniques that allow the acquisition of a proton spectrum per voxel with appropriate post-processing. This approach has been shown to work in fatty tissue and in bone marrow, using the fat proton signal as a reference, and in the brain where *N*-acetyl-aspartate serves as a reference (16–18). However, in tissues lacking a temperature-insensitive reference component, such as tumors, absolute thermometry using spectroscopic information cannot be performed.

The method we propose for the specific purpose of hyperthermia-mediated local drug delivery from drug carrier systems is based on the notions that (a) ethylene glycol is routinely used for temperature calibration in MRS experiments, (b) this calibration is based on the known temperature-dependent difference between the chemical shift of the hydroxyl and the ethylene group in ethylene glycol, and (c) the majority of the clinically relevant liposome formulations contain polyethylene glycol (PEG) (19).

In this paper, we demonstrate that PEGylated liposomes can provide a temperature-insensitive reference resonance for performing time-resolved absolute MR thermometry. Therefore, this method will be useful for hyperthermia-mediated drug delivery, as PEGylated liposomes in these experiments will serve not only as a tumor-targeted and triggerable drug delivery device, but also as an accurate nano-thermometer. In addition, we extend the idea of using drug delivery systems for providing a temperature-insensitive reference resonance for absolute thermometry to polymeric micelles and *N*-(2-hydroxypropyl)-methacrylamide (HPMA) copolymers.

2. RESULTS

2.1. Temperature-Dependence of the Frequency Difference Between the Resonances of the Protons in Water and the Protons in Peg

Figure 1(a) shows the ^1H -NMR spectra of 5 mol% DSPE-PEG-5000 liposome formulation in serum at 11 different temperatures, in

the range of 16–63°C. A temperature-dependent chemical shift of the proton resonance of water can clearly be observed, whereas the temperature-dependent chemical shift of the protons in the ethylene oxide groups (OCH_2) of PEG was found to be negligible. In Fig. 1(b) the frequency difference between the resonances of the protons in the hydroxyl group in water and that of the protons in the ethylene oxide group in PEG is plotted as function of the temperature. We observed a linear temperature-dependency of the frequency difference between the two corresponding resonances. The temperature coefficient of the chemical shift of the hydroxyl groups of water (i.e. the slope of the curve) corresponded to $-0.0093 \text{ ppm}/^\circ\text{C}$ (confidence interval, CI: -0.00942 ; -0.00920), which is in agreement with the values reported in the literature (20,21).

2.2. Relationship Total Amount of PEG and MR Signal

The integrated and normalized NMR signals of the protons of the ethylene oxide group of PEG as function of the PEG concentration (mg/ml) for the dilution series of the different liposome formulations are shown in Fig. 2. For each liposome formulation a linear relationship between the amount of PEG incorporated and the normalized magnitude of the integrated MR signal was observed. Doubling the amount of PEGylated DSPE caused

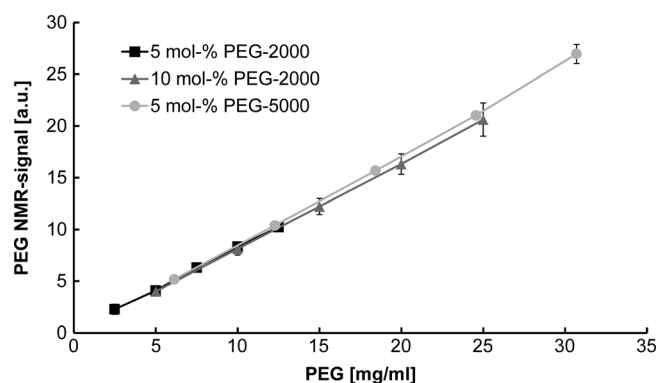


Figure 2. The area underneath the PEG NMR signal peak correlates linearly with the concentration of PEG for all three liposome formulations. To compare the MR signal of the different samples a known amount of DSS was added to each sample. The area underneath the methyl peak of DSS, resonating at 0 ppm in the proton spectrum, was used as the reference integral.

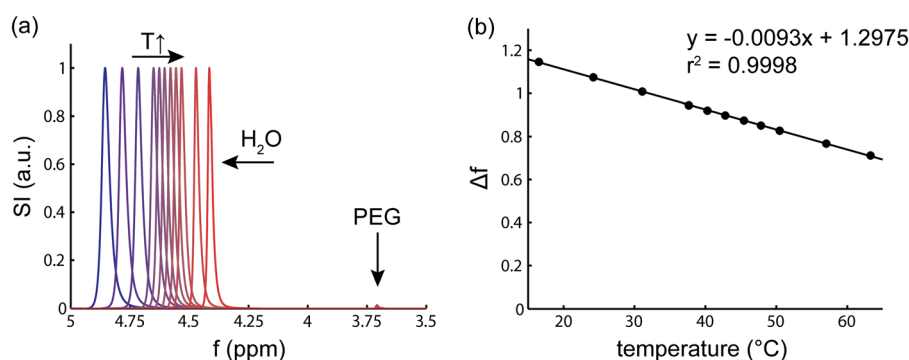


Figure 1. Proof of principle for time-resolved absolute MR thermometry using PEGylated liposomes: (a) ^1H -NMR spectra of the 5% PEG-5000 liposome in serum were recorded at 11 different calibrated temperatures. The proton resonance of water decreases with increasing temperature, whereas the proton resonance of the ethylene oxide group in PEG is constant. (b) The frequency difference between the resonances of the protons in the hydroxyl group in water and that of the protons in the ethylene oxide group in PEG is linear with temperature.

a 2-fold increase of the MR signal and replacing PEG-2000 polymers by PEG-5000 polymers increased the MR-signal by a factor of 2.5.

2.3. Influence of Environment on Temperature Coefficient

The temperature coefficient did not change under physiological salt conditions and thus remained at -0.0093 ppm/°C (CI: -0.00946 ; -0.00919). In Fig. 3, the proton resonance of water, the proton resonance of ethylene oxide group in PEG and the frequency difference between these two resonances are plotted as function of pH for 10 mol% DSPE-PEG-2000 liposomes. No change in resonances was observed as function of pH.

2.4. MR Temperature Mapping

The top row in Fig. 4 shows three temperature maps obtained using the multigradient echo (mGE) MR imaging sequence and 10 mol% DSPE-PEG-2000 containing liposomes at different temperatures. The actual temperature of the sample was verified with a calibrated fiber optic thermometer. As exemplified by the graph in Fig. 4, the average temperature of the sample measured with mGE MR imaging corresponded very well with the actual temperature ($r^2 = 0.9988$).

2.5. Detection Limits

Figure 5(a) shows a boxplot of the temperature difference between the temperature measured with mGE MRI and the temperature measured with an optical probe in 5 mol% DSPE-PEG-5000 liposome solution as function of the liposome concentration, and thus also as function of the PEG concentration. At PEG concentrations below 9.3 mg/ml the spread of the temperature differences increased; however, the median temperature differences showed little variation. This implies that the temperature measured with the mGE technique correlates well, within a range of -2.37 – 0.79 °C, with the temperature measured with the optic fiber. In Fig. 5(b) a single-voxel ^1H -NMR spectrum, obtained at 3 T, of a PEG-2000 (0.78 mg/ml) solution is shown. Furthermore, choline was added to the PEG solution at physiological concentration (5 mM). The PEG-2000 peak at 3.7 ppm is still

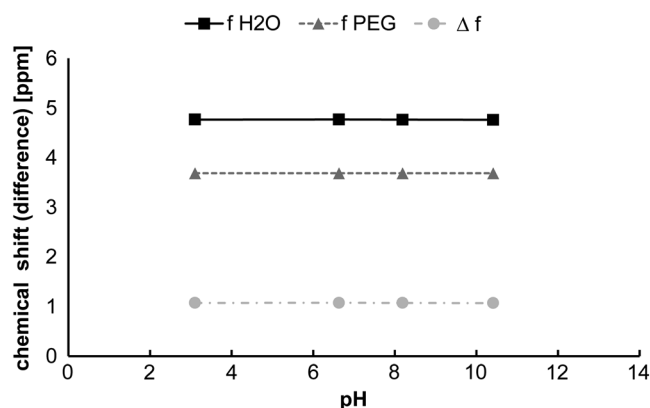


Figure 3. Influence of pH on the frequency difference between the proton resonance of water and the proton resonance of ethylene oxide group in PEG at a fixed temperature of 24.2 °C. The pH of the solution was adjusted by stepwise addition of small aliquots of a 1 M NaOH stock solution to the 10 mol% DSPE-PEG-2000 liposome solution at pH 3. Before placing the sample in the NMR spectrometer the pH was measured with a calibrated pH meter.

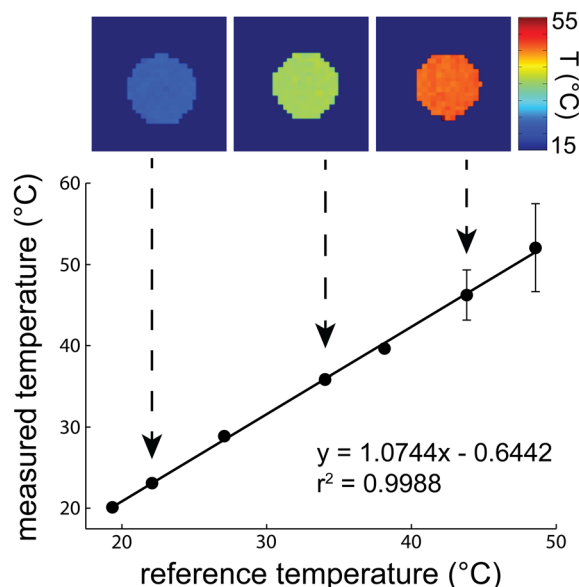


Figure 4. Temperature mapping using mGE sequence. On top three different temperature maps of the liposome solution are shown. The graph shows a high correlation between the reference temperature measured with a calibrated optical probe and the temperature measured with MRI (averaged plus standard deviation complete temperature map).

clearly detectable at this concentration and does not overlap with the choline peak at 3.2 ppm.

2.6. Proof-of-Principle for Absolute Temperature Mapping Using Polymeric Micelles and HPMA Polymers

PEG may also serve as reference in micelles based on mPEG-HPMA block polymers. However, in HPMA polymers PEG is absent; therefore other chemical groups that are present in HPMA polymers were evaluated as temperature-independent references. ^1H NMR spectra of polymeric micelles and HPMA copolymers are shown in Fig. 6a–d (only four out of 11 temperatures are shown). In the micelle samples as well as the pHMA samples, a temperature-dependent chemical shift of the proton resonance of water can clearly be observed. However, the chemical shift of the protons in the ethylene oxide groups of PEG (3.6 ppm) and of the protons in the pHPMA-Lac₂ main chain (2.5 ppm) were found to be temperature-independent (Fig. 6a and c). The same temperature-independent proton resonance was observed for different groups of the HPMA copolymer such as the methyl group (1.2 ppm), methylene groups (1.8 and 2.9 ppm) and methine group (3.9 ppm) (Fig. 6b and d).

In Fig. 6(e), the frequency difference between the resonances of the protons in the hydroxyl group in water and that of the protons in the ethylene oxide group in PEG is plotted as function of the temperature. In Fig. 6(f), the frequency difference between the resonances of the protons in the hydroxyl group in water and that of the protons in the methyl group in the HPMA copolymer is plotted as function of the temperature. In both cases, we observed a linear temperature-dependency of the frequency difference between the two corresponding resonances. The temperature coefficient of the chemical shift of the hydroxyl groups of water (i.e. the slope of the curve) corresponded to -0.0096 ppm/°C (CI: -0.00974 ; -0.00939) and to -0.0098 ppm/°C (CI: -0.00992 ; -0.00968) for polymeric micelles and HPMA copolymers, respectively. These values are very close to the value

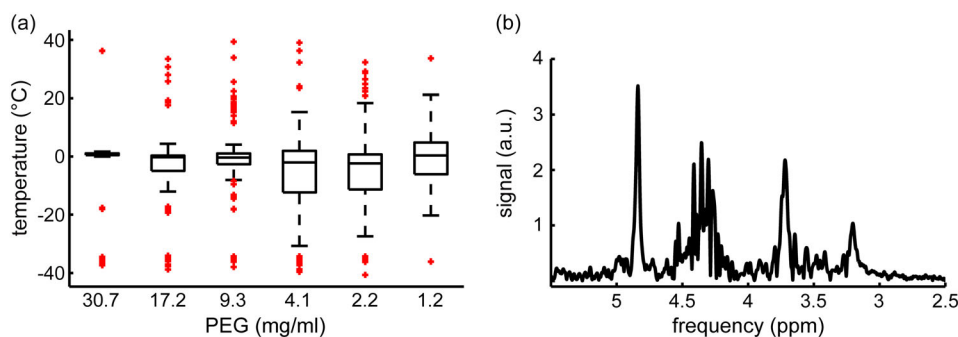


Figure 5. Boxplot of the temperature difference distribution measured in 5 mol% DSPE-PEG-5000 liposome solution, using mGE MR imaging at 3 T, as function of the liposome concentration (a). Single voxel ¹H-NMR spectrum, obtained at 3 T, of a PEG-2000 (0.78 mg/ml) solution. Choline was added to the PEG solution at physiological concentration (5 mM). The PEG-2000 and choline peaks are observable at 3.7 and 3.2 ppm, respectively.

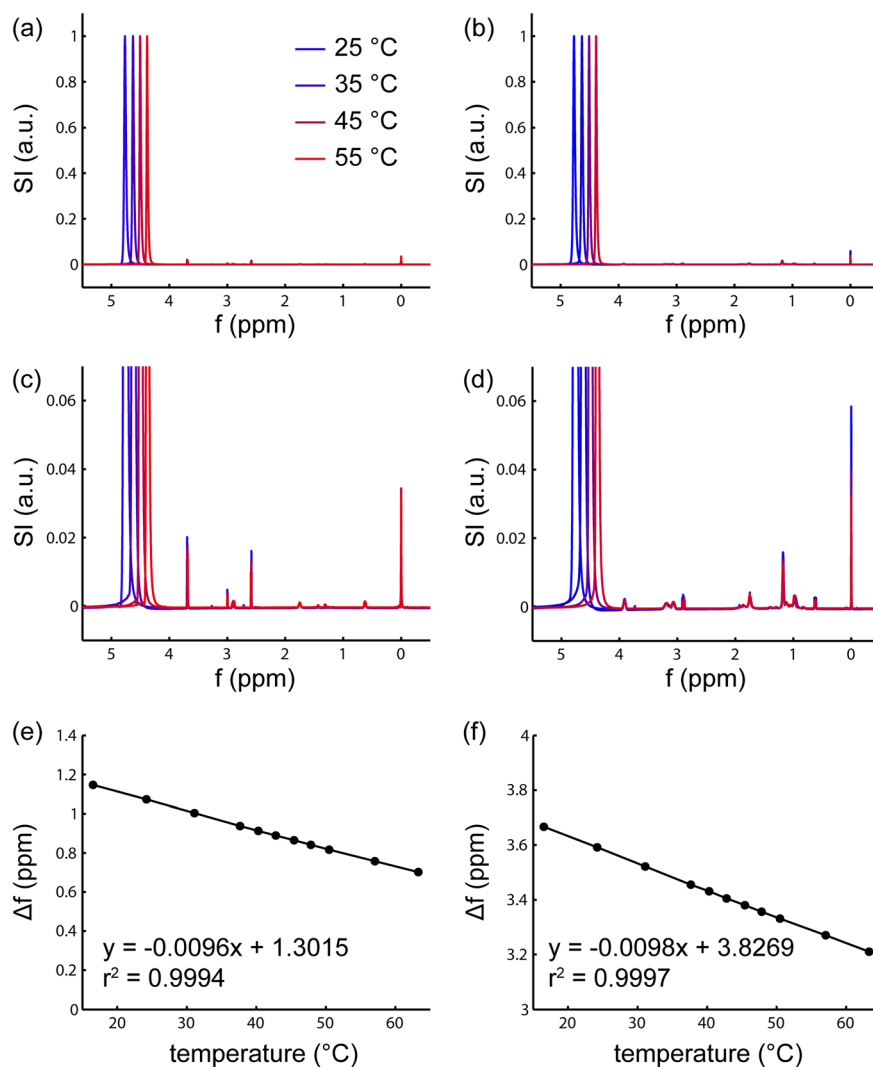


Figure 6. Prof of principle for time-resolved absolute MR thermometry using polymeric mPEG-bp(HPMAmlac₂) micelles (a, c, e) and HPMA polymers (b, d, f): (a, b) ¹H-NMR spectra of polymeric micelles and HPMA polymers shown at four different calibrated temperatures; (c, d) zoom of the ¹H-NMR spectra of polymeric micelles and HPMA polymers at four different temperatures showing that the proton resonance of water decreases with increasing temperature, whereas the proton resonance of several reference groups is constant. (e) The frequency difference between the resonances of the protons in the hydroxyl group in water and that of the protons in the ethylene oxide group in PEG is linear with temperature. (f) The frequency difference between the resonances of the protons in the hydroxyl group in water and that of the protons in the methyl group in the HPMA copolymer is linear with temperature. All experiments were performed on the 600 MHz Bruker system.

we found for temperature coefficient using liposomes, and in agreement with values reported for water in various tissue types in the literature.

3. DISCUSSION

Our results demonstrate that the chemical shift of nuclear magnetic resonances in different groups in drug delivery systems such as liposomes, polymeric micelles and HMPA copolymers can be used to provide a temperature-independent reference frequency for time-resolved absolute MR thermometry in water-rich environments like tumor tissue. Therefore this method can provide a solution for absolute thermometry in tissues lacking a temperature-insensitive reference component, especially in drug delivery applications, where the liposomes, micelles and HPMA copolymers can now act not only as a (hyperthermia-responsive) drug delivery systems, but also as accurate nano-thermometers.

Drug delivery systems such as liposomes have been used before for monitoring absolute temperature (22,23). However, in these studies the liposomes only provided information about exceeding a certain threshold temperature, by detecting contrast changes in dynamic MR images owing to the leakage of the loaded contrast agent. In addition, the contrast release is irreversible. Very recently, a new drug delivery system (hydrogel-lipid-hybrid nanoparticulate) with two threshold temperatures (i.e. lower bound for efficacy and upper bound for safety) was developed, which has several advantages compared with the temperature-sensitive liposomes, but still does not offer thermometry using a continuous temperature scale (24). Furthermore, MR thermometry based on drug delivery systems as described in these papers is dependent on the concentration of the drug delivery system in the target tissue, which can be very heterogeneous and temperature-dependent in tumor tissue (25,26). The thermometry method presented in this paper only depends on the frequency difference between two resonances and is therefore concentration-independent, provided that the water and reference resonances can be measured with a sufficiently high signal-to-noise ratio. For liposomes, the signal-to-noise ratio of the reference peak was shown to depend linearly on the concentration of PEG, suggesting that the highest possible incorporation of PEG molecules will give the most precise MR thermometry results.

As discussed above, the noninvasive character of MRI allows using the PEGylated liposomes for repeated measurements, providing means for time-resolved absolute temperature mapping. In our proof-of-principle experiments the temporal resolution was about 1 min. By fine-tuning of the scan parameters for a specific application, the temporal resolution may be reduced by a factor of 4, although it will probably never reach the same temporal resolution as the PRF method (~1 s). Still, the method described in this study can provide absolute temperature maps that serve as calibration temperature for PRF measurements or for mapping processes in which the temperature evolution is slow.

At lower liposome concentrations, and thus lower PEG concentrations, the precision of the mGE-based thermometry method decreases; however, the accuracy shows little variation. Alternatively, ^1H NMR spectroscopy can be used to measure temperature in a single voxel. Our experiments showed that NMR spectroscopy allows the detection of the PEG-signal at least

up to 32 times dilution of the initial concentration of the prepared 10 mol% PEG-2000 liposomes. According to the literature, only ~3% of the injected dose of nanocarriers accumulates in tumor tissue, depending on tumor type and tumor size, among others (27,28). Based on these numbers we might conclude that the proposed thermometry method has *in vivo* potential.

Both methods (mGE and spectroscopy) are not hampered, to a very high level of precision, by field off sets because both resonances (water and PEG) will shift with the same frequency and therefore the measured frequency difference will not change. In contrast, both methods will be hampered in case of overlap between the peak of PEG and the peak of other metabolites. However, the peak of the nearest metabolite (choline) does not overlap with the PEG-peak. When required, (partial) water and/or fat suppression might also be applied depending on the application.

4. CONCLUSIONS

We here show that nanocarriers, such as PEGylated liposomes, polymeric micelles and HPMA polymers, may serve as highly versatile tools for tumor-targeted drug delivery, acting not only as a hyperthermia-responsive drug delivery systems, but also as accurate and precise nanothermometers. Whether this technique can be applied *in vivo* remains to be further investigated.

5. EXPERIMENTAL

5.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (DSPE-PEG-2000) and dipalmitoylphosphatidylcholine (DPPC) were provided as a gift from Lipoid GmbH (Germany). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-5000] (DSPE-PEG-5000) was obtained from Avanti Polar Lipids (USA). Polyethylene glycol-2000 (PEG-2000), cholesterol (Chol), 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), D_2O 99.9% and NaCl were ordered from Sigma-Aldrich (Germany).

5.2. Liposome Preparation and Characterization

Liposomes were prepared using the well-established thin-film hydration technique (29). Three different liposome formulations were prepared based on the clinically used lipid composition DPPC-cholesterol-DSPE-PEG: (1) DPPC-Chol-DSPE-PEG-2000 (molar ratio 1.85:1:0.15); (2) DPPC-Chol-DSPE-PEG-2000 (molar ratio 1.7:1:0.3); and (3) DPPC-Chol-DSPE-PEG-5000 (molar ratio 1.85:1:0.15). The total lipid concentration for all formulations was 121.5 mM. DPPC, DSPE-PEG and Chol were dissolved in ethanol in the different molar ratios. Lipid films were formed by rotary evaporation, which were dried further under a nitrogen flow for 30 min. Once hydrated, unilamellar liposomes were obtained by multiple extrusions (Lipex Extruder, Northern Lipids, Burnaby, BC, Canada) using polycarbonate filters with progressively lower pore size, starting from 400 nm down to 100 nm. The mean hydrodynamic diameter of the liposomes was determined by dynamic light scattering (Zetasizer ZS90, Malvern, UK). The diameter of all three liposome formulations was around 130 nm and they had a polydispersity index <0.11.

5.3. Micelle Preparation and Characterization

Block copolymers composed of pHPMAm-Lac₂ as thermosensitive block and mPEG5000 as hydrophilic block were synthesized by radical polymerization using (mPEG5000)2-ABCPA as macroinitiator (ratio of monomer–initiator was 150:1 mol/mol) (30). Polymeric micelles were formed via the ‘rapid heating’ procedure at pH 5 as described previously (31). In brief, mPEG-b-p(HPMAm-Lac₂) was dissolved in ammonium acetate buffer (pH 5, 120 mM) at an initial concentration 20 mg/ml and stored overnight at 4 °C. Next, the solution was incubated at 0 °C in glass vials for at least 15 min and subsequently rapidly heated to 50 °C in a water bath under vigorous shaking for 1 min. Finally, the micelles were filtered through a 0.45 µm filter. The micelles were prepared the day before the NMR experiments and stored at room temperature. The weight- and number-average molecular weights (M_w and M_n , respectively) and the polydispersity (M_w/M_n) of the polymers were determined by gel permeation chromatography and corresponded to 36.4 kDa, 26 kDa and 1.4, respectively. The average size and size distribution of the micelles were determined with dynamic light scattering and corresponded to 90.0 nm and 0.03, respectively.

5.4. HPMA Copolymer Preparation and Characterization

The HPMA-based polymer used in this study was synthesized as described previously (32). Briefly, HPMA was prepared by the reaction of methacryloyl chloride with 1-aminopropan-2-ol in dichloromethane. *N*-Methacryloyl tyrosinamide (routinely incorporated in HPMA-based copolymers in our laboratories; to allow for radiolabeling) was prepared by the reaction of methacryloyl chloride with tyrosinamide in distilled water. Poly(HPMA-co-MA-TyrNH₂) was then synthesized by solution radical copolymerization of the monomers HPMA and MA-TyrNH₂ in methanol. M_w , M_n and M_w/M_n were determined by size exclusion chromatography on an Äkta Explorer (Amersham Biosciences). 0.3 M Sodium acetate buffer (pH 6.5) containing 0.5 g/l sodium azide was used as the mobile phase. The average molecular weight of the polymer was 64.5 kDa, and its polydispersity was 1.2.

5.5. NMR Spectroscopy

One-dimensional ¹H-NMR spectra were acquired on a Bruker Avancell Ultrashield 600 MHz NMR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) running Bruker Topspin 2.1. Per spectrum 16 measurements were accumulated and each series was preceded by four dummy repetitions to enter steady-state conditions. The relaxation delay between measurements was set to 6 s in order to have complete relaxation of the signal, the ¹H pulse angle used was 90°. Final 1D proton spectra were analyzed using MestRe-C 4.9.9.6 (Mestrelab Research, Santiago de Compostela, Spain). A standard Bruker BVT-1000 variable temperature unit was used to regulate the temperature of the air stream directed into the NMR probe. Temperature calibration was performed prior to the experiments using a dedicated temperature calibration sample (Bruker, 80% glycol DMSO-d₆) for the range of temperatures that was also used in the actual experiments.

Furthermore a couple of ¹H NMR spectroscopy experiments were performed on a clinical 3 T scanner (Achieva, Philips Healthcare, Best, The Netherlands). A single voxel chemical shift selective sequence with water suppression was performed on a volume of 1 × 1 × 1 cm³, and was planned in the center of the

liposome solution. Relevant scan parameters were: repetition time, 2500 ms; sampling interval, 0.488 ms; bandwidth, 2048 Hz; number of signal averages, 48.

5.6. MR Imaging

For time-resolved absolute MR temperature mapping on a clinical 3 T scanner (Achieva, Philips Healthcare, Best, The Netherlands), spectroscopic data was acquired at high spatial and temporal resolution using a mGE MR imaging sequence, as described by Sprinkhuizen *et al.* (18). Relevant scan parameters were: repetition time *TR*, 500 ms; first echo time *TE*₁, 3.5 ms; echo spacing, ΔTE , 3.5 ms; 32 echoes; $\alpha = 60^\circ$; acquisition voxel size, 2 × 2 × 8 mm³; number of signal averages, 1; dynamic scan duration, 65.5 s. Partial water suppression was performed using spectral selection attenuated inversion recovery in order to obtain comparable signal levels from water and PEG, which is necessary for this method.

After acquisition of the time-series of the 32 echo signals the resonance frequency difference (Δf) was found by fitting the magnitude signals of the echoes in each voxel to the signal model as described by Sprinkhuizen *et al.* (18) using in-house developed Matlab software (7.12, The MathWorks Inc., Natick, MA, USA, 2000). Subsequently the absolute temperature (in °C) was calculated from the frequency difference using:

$$T = \left(\frac{\Delta f - CS_{ref}}{\alpha} \right) + T_{ref} \quad (1)$$

in which α is the temperature coefficient of the chemical shift of protons in water (−0.01 ppm/°C) and CS_{ref} is the chemical shift between the protons in water and the protons in the ethylene oxide groups of PEG (= 0.9527 ppm) at a reference temperature T_{ref} (= 37.7 °C).

5.7. Temperature Dependence of Proton Resonance of PEG

To investigate the temperature dependence of the proton resonance of the hydroxyl group in water and of the proton resonance of the ethylene oxide groups in PEG, 1D ¹H-NMR spectra were recorded at 11 different temperatures ranging between 16 and 63 °C. Per temperature, the proton resonance of the ethylene oxide group in PEG and the proton resonance of the hydroxyl group in water were determined from the acquired spectrum by the peak picking algorithm as implemented in MestRe-C. The samples were prepared by mixing 450 µl of the liposome solution, 50 µl D₂O (for field locking) and a known amount of DSS (internal chemical shift reference) in a regular Wilmad 5 mm NMR sample tube, which was subsequently placed in the NMR spectrometer. To ensure sufficient time to get a stable and constant temperature, a temperature equilibration period of 15 min was allowed at each temperature step.

5.8. Relationship Between NMR Signal and PEG Concentration

In order to determine which liposome formulation can be best used for thermometry the relationship between the amount of PEG and the NMR signal was measured of dilution series of the three liposome formulations (i.e. 5 mol% DSPE-PEG-2000, 10 mol % DSPE-PEG-2000 and 5 mol% DSPE-PEG-5000). The dilution series were prepared by diluting the different liposome stock solutions

[i.e. 90% (v/v) liposomes in PBS and 10% (v/v) D₂O for field locking] with PBS [10% (v/v) D₂O]. A known amount of DSS was added to stock solution and used as internal chemical shift reference and for normalization. The final DSS concentration per sample was in the range 73–84 mM. For each sample in the dilution series the areas under the PEG and DSS peaks were determined by signal integration. The area under the DSS peak was used to normalize the spectrum for coil sensitivity. The sample was transferred to a regular Wilmad 5-mm NMR sample tube and placed in the spectrometer. We waited for 15 min to let the temperature stabilize at 24.1°C before starting the measurement. The experiments were performed twice for each liposome formulation.

5.9. Influence Environment on Temperature Coefficient

Preferably, the proposed method should not depend on the physiological salt conditions and local pH. Therefore, the frequency difference between the resonances of the protons in the hydroxyl group in water and that of the protons in the ethylene oxide group in PEG was also measured under physiological salt conditions (0.9% NaCl solution) and at various pH values in the range pH 3–10 at a fixed temperature of 24.2 °C for 10% PEG-2000 liposomes. NaOH (1 M) was added to the liposome samples to change the pH. The pH was verified with a pH-probe (Mettler Toledo) before the sample were transferred to a regular Wilmad 5 mm NMR sample tube and placed in the spectrometer.

5.10. Absolute Temperature Mapping

In order to show that liposomes can be used for time-resolved absolute temperature mapping, we performed a proof-of-principle experiment. The experimental set-up consisted of a 25 ml boiling flask filled with liposome solution suspended in a 2000 ml beaker filled with manganese (added in order to shorten relaxation times of water) doped water. The water in the beaker plus the liposome solution in the boiling flask were heated up to the desired temperature outside the MR scanner using a warm water bath. Subsequently, the complete set-up was transferred into the MR bore and calibrated fiber optic temperature probes (Luxtron) were inserted in the water and liposome solution. MR thermometry was performed, using the mGE imaging sequence, only when both the water bath and the liposome solution were in thermal equilibrium.

5.11. Detection Limits

In order to investigate the detection limits of the mGE method and ¹H NMR spectroscopy at 3 T, dilution series of 5 mol% DSPE-PEG-5000 liposomes and PEG-2000 were prepared, respectively. For the dilution series of the liposomes the PEG concentrations were in the range of 1.2–30.7 mg/ml. For the PEG-2000 dilution series the PEG concentrations were in the range of 0.78–25.0 mg/ml. Furthermore, 5 mM choline was added to each sample. Next, a 25 ml boiling flask was filled with a liposome or PEG-2000 sample, suspended in a 2000 ml beaker filled with manganese doped water at room temperature and MR imaging/spectroscopy was performed as described above. The actual temperature of the sample was also measured with a calibrated fiber optic temperature probe.

5.12. Proof-Of-Principle Absolute Temperature Mapping Using Polymeric Micelles and H₂MA Copolymers

Block copolymers composed of pHPMAm-Lac₂ and mPEG-5000 form the building blocks for polymeric micelles. To investigate if the mPEG-5000 block also can be used as temperature insensitive reference resonance for performing absolute thermometry, 1D ¹H-NMR spectra of polymeric micelles were recorded at 11 different temperatures ranging between 16 and 63 °C. The same temperature series was also acquired for HPMA copolymers. HPMA polymers do not contain PEG; however, the repeating structural unit of HPMA copolymers contains several chemical groups that may serve as temperature-insensitive reference resonance. The micelle and pHPMA samples were prepared by mixing 450 µl of the micelle/HPMA solution, 50 µl D₂O and a known amount DSS in a regular Wilmad 5 mm NMR sample tube, which was subsequently placed in the NMR spectrometer. The acquired ¹H NMR spectra were treated as described above for liposomes.

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